THE JOURNAL OF PROSTHETIC DENTISTRY

RESEARCH AND EDUCATION

# Biocompatibility of 4-META/MMA-TBB resin used as a dental luting agent

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## ABSTRACT

**Statement of problem.** The bonding and biological properties of currently used luting/cementing materials need to be improved. 4-Acryloyloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butylborane (4-META/MMA-TBB) resin is primarily used for splinting mobile teeth or treating fractured teeth. It undergoes moisture-resistant polymerization and bonds strongly to dentin and metals.

**Purpose.** The purpose of this in vitro study was to compare the biological and biochemical properties META/MMA-TBB resin with those of conventional polymethyl methacrylate (PMMA)-MMA resin and other currently used luting materials in order to determine whether it may be a viable dental luting agent.

**Material and methods.** The degree of polymerization of 4-META/MMA-TBB resin, PMMA-MMA autopolymerizing resin, 10-methacryloyloxydecyl dihydrogen phosphate-dimethacrylate (MDP-DMA) adhesive resin, and a glass ionomer cement was measured by Fourier-transformed infrared spectroscopy. Free radical production during setting was evaluated by electron spin resonance (ESR) spectroscopy. Rat dental pulp cells cultured on these materials were examined for cell viability, attachment, proliferation, and functional phenotype.

**Results.** The degree of polymerization of 4-META/MMA-TBB resin was 82% thirty minutes after preparation, compared to 66% for PMMA-MMA autopolymerizing resin. ESR spectroscopy revealed free radical production from 4-META/MMA-TBB resin and glass ionomer cement was equivalent 24 hours after preparation, with no spike in radical generation observed. In contrast, free radical production from PMMA-MMA and MDP-DMA adhesive resins was rapid and sustained and 10 to 20 times greater than that from 4-META/MMA-TBB. The percentage of viable dental pulp cells 24 hours after seeding was considerably higher on MDP-DMA and 4-META/MMA-TBB resin than on glass ionomer cement. Cell number, proliferation, and alkaline phosphatase activity were highest on 4-META/MMA-TBB resin and lowest on the glass ionomer cement.

**Conclusions.** 4-META/MMA-TBB resin is at least as biocompatible, and perhaps even more biocompatible, than other current luting materials, with fast, favorable, and nontoxic polymerization properties. Further in vivo and human studies of 4-META/MMA-TBB resin as a dental luting agent are warranted. (J Prosthet Dent 2015;114:114-121)

Developing more biocompatible materials while maintaining or improving their mechanical properties remains a challenge in restorative dentistry. Acrylic-based autopolymerizing resin, which primarily consists of solid prepolymerized polymethyl methacrylate (PMMA) and liquid methyl methacrylate (MMA), is frequently and extensively used in dental practice for the fabrication of interim restorations and in denture repair. Despite its easy practical use and acceptable hardening behavior and mechanical properties, PMMA/MMA resin should be avoided for direct intraoral use because of low biocompatibility, or, in other words, its high cytotoxicity.[1-3] PMMA/MMA resin causes extensive adverse effects at both the cell and tissue level, with evidence of induction of apoptosis, mutagenesis, inhibition of differentiation, and necrotic and fibrotic reactive changes in the surrounding tissue.[1,4-13] The chemical and biological effects of PMMA/MMA resin have been of particular clinical concern when applied directly onto

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## Clinical Implications

The setting behavior of 4-acryloyloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butylborane (4-META/MMA-TBB) resin is unique: despite a very low level of free radical production, its setting is rapid and complete. These biochemical properties result in its high biocompatibility with dental pulp cells compared to other currently used luting materials. Given its moisture-resistant polymerization and strong bonding capability to both dentin and metal, 4-META/MMA-TBB resin may be suitable as a luting agent.

prepared teeth, for instance, as a dental luting cement, because of cytotoxic effects on the dental pulp cells and tissue through the exposed dental tubules.[14,15]

Due in part to these concerns, 4-META/MMA-TBB resin was developed by adding 4-methacryloyloxylethyl trimellitate anhydride (4-META) to MMA, with tri-n-butyl borane (TBB) as a polymerization initiator (Fig. 1).[16-19] The molecular structure of 4-META was originally designed for preparation by condensing 2-hydroxyethyl methacrylate and trimellitic anhydride chloride in the presence of pyridine to remove hydrogen chloride.[16,19] When added at 1% to 5% concentration to conventional acrylic resin, it forms hydrophilic and hydrophobic methacrylate (Fig. 1) and produces new and enhanced dentin bonding, termed the hybrid layer.[19-21] The hybrid layer is caused by the penetration of the monomer into dentin and allows for the formation of a transitional zone of resin-reinforced dentin. The presence of 4-META also enhances bond strength to metals. Because of its polarity, 4-META forms a hydrogen bond with the oxygen or hydroxyl group of an oxide layer on metal surfaces. These significantly enhanced mechanical properties of 4-META/MMA-TBB resin over conventional PMMA resin broaden its clinical applications,[16,20-25] and the resin has been used to splint mobile teeth, treat fractured teeth, and seal dental root ends.[16,26]

This study focused on a further advantages of 4-META/MMA-TBB resin, namely moisture-resistant polymerization and favorable biocompatibility, which have previously been exploited for healthy mucosal healing and bone bonding in experimental animal models.[27-30] Because cemented dental restorations are always contaminated with moisture from saliva and fluid from the gingival sulcus, tooth-to-cement bonding and margin sealing around the restorations are compromised. 4-META/MMA-TBB resin, which is moisture resistant, may provide a solution to overcome these problems. In contrast to traditional luting agents, which can cause chemically induced and irreversible pulp chamber reactions and compromise clinical outcomes with acute and chronic pain and inflammation, pulp tissue necrosis, and periapical lesions, 4-META/MMA-TBB resin may act as a biocompatible luting agent. However, the exact biocompatibility of 4-META/MMA-TBB resin with dental pulp cells, in contrast with existing materials, is unknown.

This study tests 2 hypotheses: that the biochemical properties of 4-META/MMA-TBB resin are better than conventional PMMA resin and representative, currently used dental luting materials, and that 4-META/MMA-TBB resin shows at least equivalent, or perhaps more, biocompatibility than existing luting materials. Therefore, the objective of this study was to examine the polymerizing behavior of 4-META/MMA-TBB resin (Super-bond C&B; Sun Medical), specifically the degree of polymerization and free radical generation in comparison with a conventional PMMA autopolymerizing resin (Unifast II; GC America Inc) and to compare the viability and function of dental pulp cells cultured on a 4-META/MMA-TBB resin, glass ionomer cement (Fuji I; GC America Inc), and 10-methacryloyloxydecyl dihydrogen phosphate-dimethacrylate (MDP-DMA) (Panavia F. 2.0; Kuraray America Inc) adhesive resin and cement. The high biocompatibility of glass ionomer cement and MDP-DMA adhesive resin cement has been reported.[31-37]

## MATERIAL AND METHODS

### Biochemical characterization of 4-META/MMA-TBB resin

To identify the potential biochemical advantages of 4-META/MMA-TBB resin over the currently used dental luting materials, 4-META/MMA-TBB resin (Super-bond C&B; Sun Medical), glass ionomer cement (Fuji I; GC America Inc), and MDP-DMA adhesive resin cement (Panavia F. 2.0; Kuraray America Inc) were prepared according to the manufacturers' instructions. Because the MDP-DMA used in this study was based on the dual-polymerization mechanism of chemical and light polymerization, it was irradiated with visible light (450 to 490 nm) for 20 seconds by using a 1200 mW/cm$^2$ light-emitting diode instrument (Blue Life LED-320 Cordless; Microtech). In addition, to establish how 4-META/MMA-TBB resin is different from the conventional PMMA-based resin, PMMA autopolymerizing resin (Unifast II; GC America Inc) was prepared.

The primary objective of this study was to determine the biocompatibility of 4-META/MMA-TBB resin in comparison with other currently used materials. However, to confirm and accurately interpret biological results, this study rationalized that biochemical characterization is also necessary for these materials. Although methodological restriction may apply because the materials used in this study vary in the base material and setting mechanism, a set of 2 different biochemical

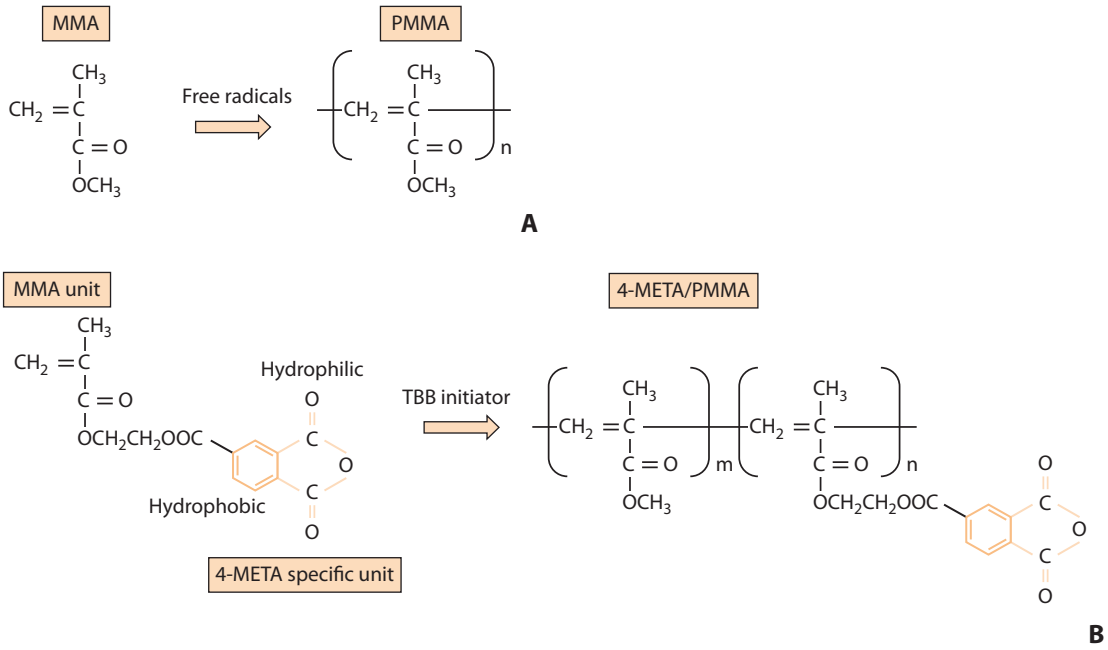


**Figure 1.** Structure of conventional PMMA/MMA resin (A) and 4-META/MMA-TBB resin (B).

characterizations was designed to evaluate the polymerization behavior of the materials.

First, the degree of polymerization was evaluated with Fourier-transformed infrared spectroscopy (FT-IR) (Spectrum100; PerkinElmer). By analyzing FT-IR spectra, polymerization behavior can be characterized.[38,39] This study evaluated the degree of polymerization based on the increasing number of repeat units of the polymer chain during polymerization. The ratio of 1720 $cm^{-1}$ (C=O) absorbance and 1640 $cm^{-1}$ (C=C) was measured at the commencement of power/liquid mixing and 30 minutes after the mixing, and the degree of polymerization was calculated as the reduction rate of 1640 $cm^{-1}$ (C=C) absorbance. FT-IR was conducted for 4-META/MMA-TBB resin, MDP-DMA adhesive resin cement, and PMMA autopolymerizing resin. Because glass ionomer cement is not a polymerizing material, the degree of polymerization could not be evaluated.

Second, the generation of free radicals within the setting materials was assessed by an electron spin resonance spectroscopy (ESR), which has been developed and validated for various biomedical applications.[40-42] Specimens were examined with a JES-RE 3X, X-band spectrometer (JEOL) connected to a WIN-RAD ESR Analyzer (Radical Research) at the following settings: modulation amplitude, 0.063 mT; sweep width, 5 mT; sweep time, 1 minute; time constant, 0.03 seconds; microwave power, 8 mW; and magnetic field, 335.5 mT. The component signals in the spectra were identified and quantified as reported previously.[40] The level of polymerization radicals was measured at multiple time points up to 24 hours after specimen preparation.

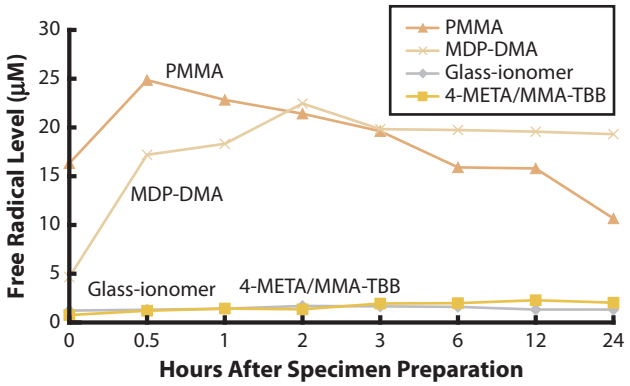


**Figure 2.** Polymerization radical generation in 4 different materials. Level of free radicals evaluated by electron spin resonance spectroscopy (ESR) was plotted from immediately after specimen preparation up to 24 hours.

## Substrate preparation for cell culture

Substrates for culturing dental pulp cells were prepared according to the manufacturers' instructions by using 3 different types of the previously mentioned dental luting products (glass ionomer cement, MDP-DMA adhesive resin cement, and 4-META/MMA-TBB resin) and spread evenly in wells of 12-well cell-culture-grade polystyrene dishes with a thickness of 0.4 mm. Cells were seeded on the substrates immediately (within 3 minutes) after each of the substrate materials was spread.

## Dental pulp cell culture

Cell culture procedure was similar to that used in the previous studies using dental pulp cells.[1-3,13,43,44] Briefly, dental pulp cells extracted from the maxillary incisors of

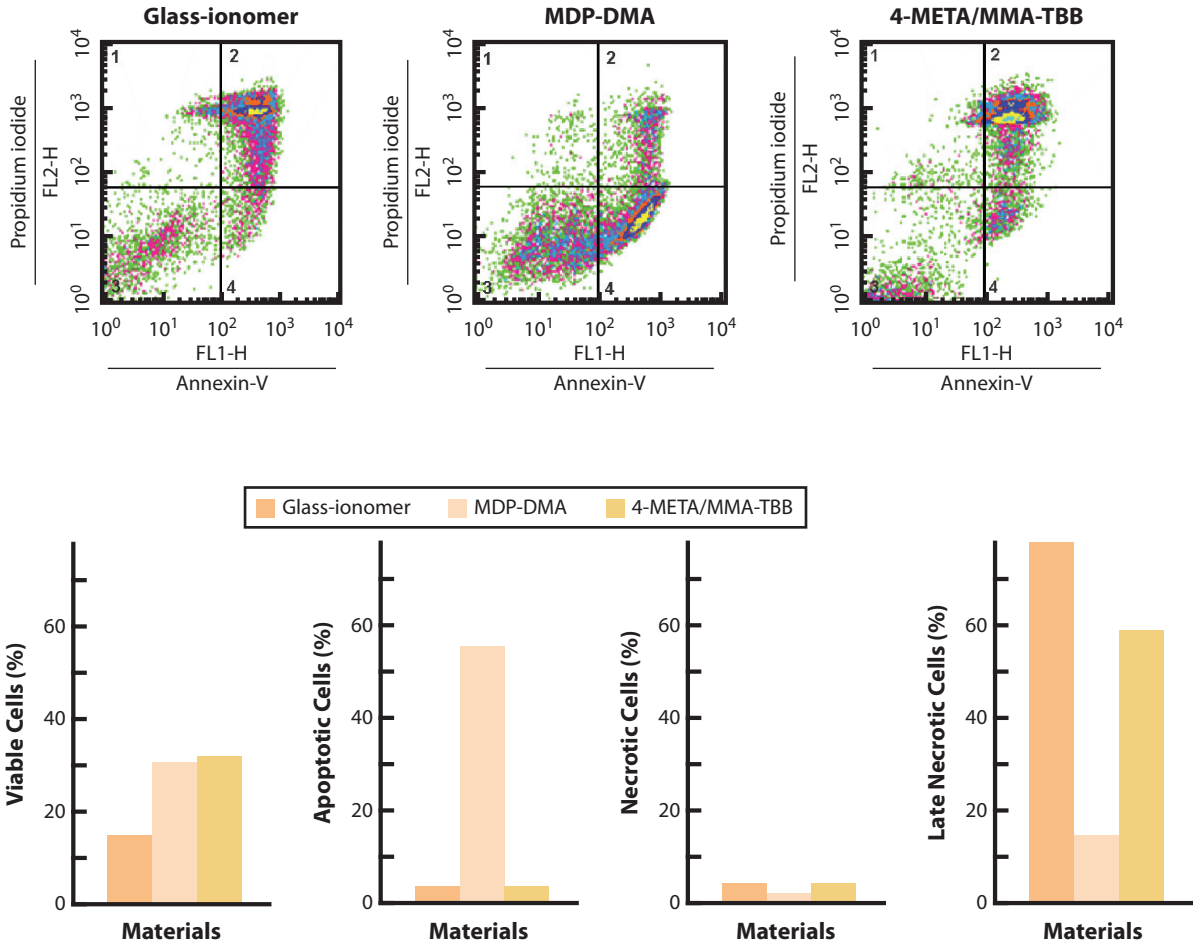


**Figure 3.** Flow cytometry-based viability/death analysis of dental pulp cells on various luting materials. Cells 24 hours after seeding on these materials were analyzed. Flow cytometric images (top) and percentages of viable cells (quadrant 3 on top images), apoptotic cells (quadrant 4), necrotic cells (quadrant 1), and late necrotic cells (quadrant 2).

8-week-old male Sprague Dawley rats were placed in alpha-modified Eagle medium supplemented with 15% fetal bovine serum, 50 μg/mL ascorbic acid, $10^{-8}$ M dexamethasone, and 10 mM Na-β-glycerophosphate, and an antibiotic-antimycotic solution containing 10 000 units/mL penicillin G sodium, 10 000 mg/mL streptomycin sulfate, and 25 mg/mL amphotericin B. Incubation was performed under humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. At 80% confluency, the cells were detached by using 0.25% trypsin-1 mM EDTA-4Na and seeded directly onto the prepared substrates at a density of $3\times10^4$ cells/$cm^2$. The culture medium was renewed every 3 days.

## Cell viability assay

A flow cytometry-based cell viability test was performed with the cells after a 24-hour incubation. The test was similar to the method used in the previous studies[3,13] and based on the flow cytometric detection of annexin V binding and propidium iodide (PI) staining (Annexin V-FITC Kit; BD Bioscience). Annexin V is known to bind to phosphatidylserine and PI to DNA when the cell membrane is dismantled. The intensity of PI staining (y-axis) was plotted against Annexin-FITC intensity (x-axis). Viable cells were observed in the lower left quadrant (annexin V negative/PI negative), apoptotic cells in the lower right quadrant (annexin V positive/PI negative), necrotic cells in the upper left quadrant (annexin V negative/PI positive), and late necrotic cells in the upper right quadrant (annexin V positive/PI positive).

## Cell attachment, density, and proliferation assays

These procedures have been routinely performed in the authors' laboratory and described in detail elsewhere.[1-3,13,43,44] Briefly, initial cell attachment was evaluated by measuring the number of cells attached to the substrates after 3 and 24 hours of incubation. Propagated cells were also quantified as cell density at day 2 of culture. Quantifications were performed through colorimetry with a tetrazolium salt (WST-1) (Roche Applied Science). A culture well was incubated at 37°C for 4 hours with the WST-1 reagent (100 μL), and the amount of formazan produced was measured with an enzyme-linked

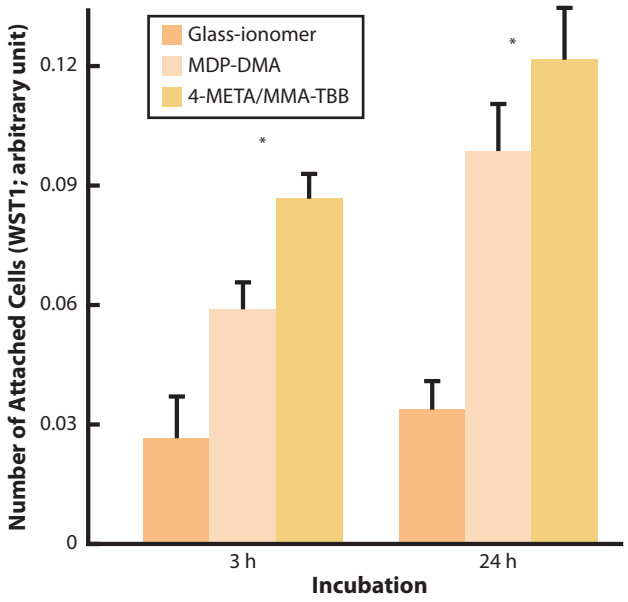


**Figure 4.** Initial-stage attachment of dental pulp cells to various luting materials. Number of attached cells after incubation for 3 and 24 hours, as evaluated by WST-1 colorimetry. Data are mean ±SD (n=3). *$P<.05$, indicating statistically significant difference among 3 different materials.

immunosorbent assay (ELISA) reader at 420 nm (Synergy HT; BioTek Instruments). The proliferative activity of cells was measured by BrdU incorporation during DNA synthesis. At day 2 of culture, a 100 mM BrdU solution (100 µL) (Roche Applied Science) was added to the culture wells and incubated for 10 hours. After trypsinizing the cells and denaturing the DNA, the cultures were incubated with anti-BrdU antibody conjugated with peroxidase for 90 minutes, then exposed to tetramethylbenzidine for color development. Absorbance was measured with an ELISA reader at 370 nm.

### Alkaline phosphatase (ALP) activity

The ALP of dental pulp cells was examined at day 7 with image-based and colorimetry-based assays. For the image analysis, cultured cells were washed twice with Hanks solution and incubated with 120 mM Tris buffer (pH 8.4) containing 0.9 mM naphthol AS-MX phosphate and 1.8 mM fast red TR for 30 minutes at 37°C. The ALP-positive area on the stained images was calculated as ([stained area/total dish area]×100) (%) with an image analyzer (ImageJ; NIH). For colorimetry, cells were rinsed with $ddH_2O$ and incubated at 37°C for 15 minutes in the presence of p-nitrophenylphosphate (250 µL) (LabAssay ATP; Wako Pure Chemicals). ALP activity was evaluated as the amount of nitrophenol released by the enzymatic reaction and measured with an ELISA reader at 405 nm.

### Statistical analysis

Three specimens were analyzed for cell culture and ESR studies (n=3). Flow cytometric evaluations were performed 3 to 5 times, and a representative data set was presented after confirming their consistency. ANOVA was used to assess differences among different substrate materials ($\alpha$=.05).

## RESULTS

FT-IR spectroscopic analysis showed that the degree of polymerization was 82% for the 4-META/MMA-TBB resin 30 minutes after the specimen preparation, while conventional PMMA resin was 66% polymerized. In particular, 4-META/MMA-TBB resin showed greatly increased polymerization to MDP-DMA adhesive resin, which was 0% polymerized. The surface of MDP-DMA adhesive resin failed to harden even after light irradiation.

ESR analyses showed that free radical production from 4-META/MMA-TBB resin was very low throughout the 24 hours of follow-up after specimen preparation, in contrast to conventional PMMA resin (Fig. 2). Free radical production from PMMA resin was 10 to 20 times greater than 4-META/MMA-TBB resin in the initial 3 hours of follow-up. Although free radical production from PMMA resin appeared to start to decrease after 3 hours, free radical production still remained 7 times greater than 4-META/MMA-TBB resin, even at 24 hours. MDP-DMA adhesive resin also showed an immediate and rapid production of free radicals after mixing, with production remaining very high and similar to PMMA resin from 0.5 to 24 hours. The level of free radical production from glass ionomer cement was as low as that from the 4-META/MMA-TBB resin.

Dental pulp cells were seeded on 4-META/MMA-TBB resin and 2 different luting materials (glass ionomer cement and MDP-DMA resin), and cell viability and function were examined 24 hours after seeding (Fig. 3). The percentage of viable dental pulp cells on MDP-DMA and 4-META/MMA-TBB resin was approximately twice that on glass ionomer cement. Cell death on 4-META/MMA-TBB resin was characterized by a low percentage of apoptotic cells and a high percentage of late apoptotic cells compared to those grown on MDP-DMA.

The number of attached dental pulp cells was significantly different among materials after 3 hours of incubation and was highest on 4-META/MMA-TBB resin, followed by MDP-DMA and glass ionomer cement (Fig. 4). Even after 24 hours of incubation, the number of attached cells remained different and was highest on 4-META/MMA-TBB resin and lowest on the glass ionomer cement. The number of attached cells did not increase between 3 and 24 hours on the glass ionomer cement.

Because cell attachment increased during the first 24 hours of culture on 4-META/MMA-TBB resin, it was next

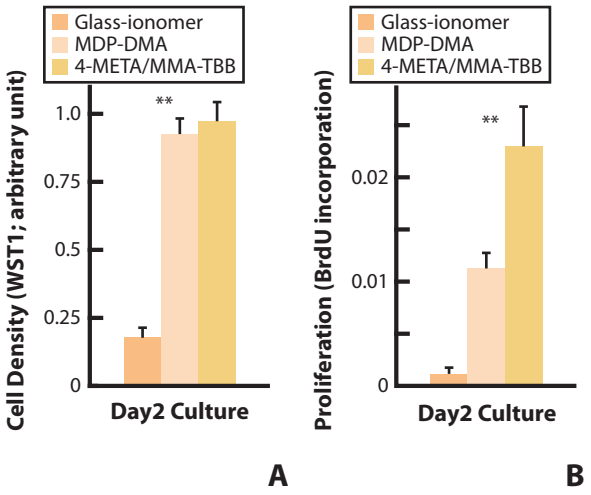


**Figure 5.** Proliferative activity of dental pulp cells on various dental restorative materials. A, Cell density at day 2 of culture, as evaluated by WST-1 colorimetry. B, BrdU incorporation into DNA evaluated at day 2 of culture. Data are mean ±SD (n=3). ***P*<.01, indicating statistically significant difference among 3 different materials.

determined whether this resulted in an increased number of cells and proliferative activity on each substrate during longer-term culture. The cell density at day 2 on 4-META/MMA -TBB resin and MDP-DMA was considerably higher than that on glass ionomer cement (Fig. 5A). No significant difference was found between the MDP-DMA and 4-META/MMA-TBB resin. Proliferation was markedly different for the 3 substrates and was highest on the 4-META/MMA-TBB resin and lowest on the glass ionomer cement (Fig. 5B).

At day 7, the ALP-positive area was highest on 4-META/MMA-TBB resin, followed by MDP-DMA and glass ionomer cement (Fig. 6A); this was corroborated by colorimetric detection of ALP (Fig. 6B), which had a similar pattern. ALP activity on 4-META/MMA-TBB resin was approximately 25 times greater than that on the glass ionomer cement and 1.8 times greater than that on MDP-DMA resin.

## DISCUSSION

This study hypothesized that 4-META/MMA-TBB resin is at least equivalent to or potentially more biocompatible than current commercially available luting agents because of the improved biochemical properties. Within the materials tested, the hypothesis has been proven positively, providing the rationale and justification for the potential use of 4-META/MMA-TBB resin as a more biologically and biochemically compatible luting agent.

Cell viability and functional assay results were consistent; cell attachment, growth, and function were substantially greater on 4-META/MMA-TBB resin than on the other 2 luting materials for all the variables tested. For instance, ALP activity of dental pulp cells cultured on 4-META/MMA-TBB resin was 1.8 times greater than those cultured on MDP-DMA and 25 times greater than on glass ionomer cement. Glass ionomer cement is reported to be minimally cytotoxic unless constituted from polymerizing molecules, such as in resin-modified glass ionomer cement.[31-34] Surprisingly, perhaps, MDP-DMA adhesive resin cement was much less cytotoxic than glass ionomer cement. The MDP-DMA used in the study, Panavia F2, has been shown to be less toxic than other resin or zinc phosphate cements,[35,36] particularly when light is used to complete polymerization.[37] It is therefore probably not appropriate to generalize that resin-based adhesive cements are less cytotoxic than glass ionomer cements; however, the 4-META/MMA-TBB resin was the least cytotoxic material tested.

The biochemical profile of the 4-META/MMA-TBB resin was consistent with the results from the biological assays. The high viability of cells during the initial stage of culture and the ALP activity during the later stages of culture can be explained by the low production of polymerization radicals by 4-META/MMA-TBB resin. The functional phenotype is important to assess because surviving cells that are exposed to toxic materials may have aberrant phenotypes resulting from impaired transcription, translation, and differentiation (or induce dedifferentiation), with consequent functional deterioration.[1,12,13] Polymerization free radicals are molecular species with unpaired electrons and by-products of polymerization and decomposition of monomer and initiators. There are 2 major mechanisms underlying the adverse effects of polymerizing resin materials.[1,3,13] The first is the direct damage on the cellular structure, such as the dismantlement of the cell membrane as a result of the reactive nature of free radicals. The second is the creation of imbalance between the oxidative stress and antioxidant redox defensive system within the cell. Free radicals infiltrated into cells produce oxidative stress. Once the oxidative stress exceeds the capacity of the cellular antioxidant redox system, it starts to induce DNA mutations and modulates the signaling pathways, leading to functional impairment and cellular apoptosis.[10,11] The present results suggest that cells that survive on 4-META/MMA-TBB resin remain healthy enough to manifest normal function, as demonstrated by the high proliferative and ALP activity. The production of free radicals was substantially lower from META/MMA-TBB resin compared to PMMA and MDP-DMA resins, and no initial spike was observed in radical generation after material preparation. The free radicals produced by META/MMA-TBB resin were as low as those from glass

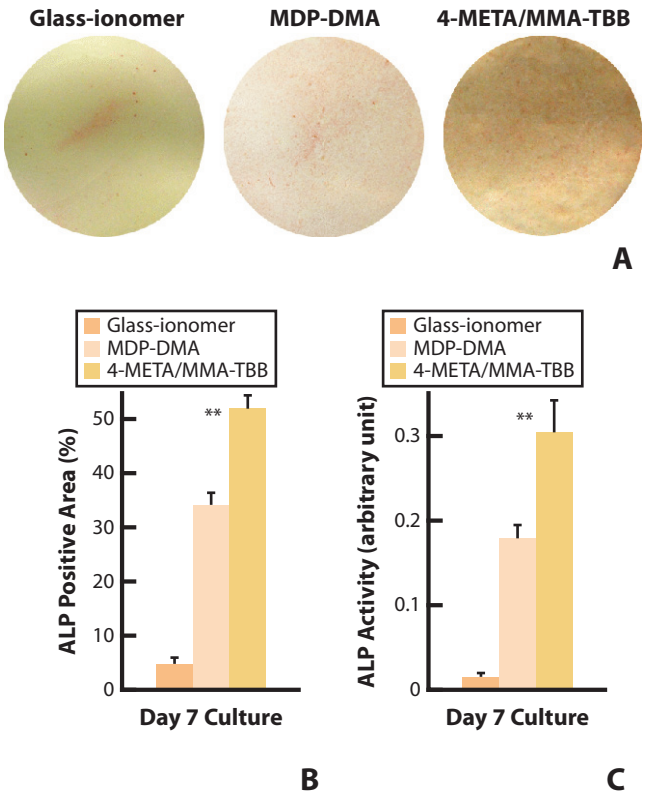


**Figure 6.** Alkaline phosphatase (ALP) activity in dental pulp cells on various luting materials. A, ALP activity evaluated by image-based densitometry. At top are images of ALP staining of dental pulp cells cultured for 7 days. B, ALP-positive area as percentage of culture area was measured with digital image analyzer and presented in histograms. C, ALP activity evaluated by colorimetry. Data are mean ±SD (n=3). $^{**}P<.01$, indicating statistically significant difference among 3 different materials.

ionomer cement. In spite of minimal free radical generation, the degree of polymerization in the 4-META/MMA-TBB was greater than that of the PMMA resin, suggesting that the amount of monomer remaining in the material after initial setting is likely to be minimal. The accelerated and more complete polymerization and minimal emission of toxins were therefore closely associated with the improved biocompatibility of the 4-META/MMA-TBB resin.

This study simulated the extreme condition of dental pulp cells in direct contact with material surfaces, which is likely to be the most challenging set of conditions for cell survival and function; they are directly exposed to remnant monomers and the reactive oxygen species released from the materials during polymerization.[2,13] However, this is a useful in vitro model that represents a clinically plausible environment, in which the chemical and biological effects of luting or restorative materials are imposed on dental pulp cells and tissue through the exposed dental tubules.[1,3,13-15] Even under these challenging conditions, many dental pulp cells survived and displayed their anticipated phenotype, such as ALP activity, on MDP-DMA and 4-META/MMA-TBB resins.

This pilot study provides essential in vitro data for the design of in vivo experiments to test the use of 4-META/MMA-TBB resin as a dental luting agent. The biological and biochemical results mutually demonstrate the potential advantages of the material. Together with 4-META/MMA-TBB resin's proven physical strength, bonding capability, ease of use, and moisture-insensitive polymerization, these biological and biochemical characteristics further emphasize the promise of this material as a luting agent.

## CONCLUSIONS

The biological and biochemical properties of 4-META/MMA-TBB resin were evaluated in vitro to assess its potential use as a dental luting agent, and the following conclusions were drawn:

1. 4-META/MMA-TBB resin underwent rapid and near complete polymerization with considerably lower free radical production than PMMA or MDP-DMA adhesive resins.
2. Free radical production from 4-META/MMA-TBB resin was as low as that from glass ionomer cement.
3. The percentage of viable dental pulp cells 24 hours after seeding was considerably higher on MDP-DMA and 4-META/MMA-TBB resin than on glass ionomer cement.
4. Cellular function parameters, such as proliferation and ALP activity, were highest on 4-META/MMA-TBB resin and lowest on glass ionomer cement.

The results demonstrating the high biocompatibility of 4-META/MMA-TBB resin added to its uniquely favorable biochemical property during polymerization.

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